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PRELIMINARY REVIEW OF THE DETERMINANTS RESPONSIBLE FOR VIRULENCE OF MICROBIOLOGICAL ORGANISMS

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This review of the knowledge of the mechanisms of pathogenicity covers research principles gradually evolved from studies done from 1937 to 1985. Five factors were found to be generally involved in microbial pathogenicity: (1) infection of the mucous surfaces (adherence) or direct host entrance by skin trauma or vectors; (2) penetration of the mucous surfaces to enter the tissue; (3) multiplication in the hosts' tissues; (4) interference with the host defense (bacteriocidins, phagocytes, complement, and immune response); and (5) damage to the host (toxins and immunopathological mechanisms) - chemical compounds, usually on the surface of the microorganism, determine this pathogenicity. There may be more than one determinant due to the multifactorial nature of pathogenicity. Each determinant must be distinguished from others, making the biochemical task complex. In studying molecular biology, using manipulation by recombinant DNA techniques and cloning, investigators may find that genes on the DNA segment code for the putative determinant, or pleitropy may
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occur, where the gene product may not be the determinant but causes the production of other cell constituents, some of which may be the true determinants. Genes will be found on chromosomes, plasmids, or phages.

Adherence (infection of the mucous surface) is the only virulence factor at the determinant stage of research in bacteria pathogenicity. For other microbes, only the multiplication of viruses in vivo was at the determinant level.

PREFACE

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PRELIMINARY REVIEW OF THE DETERMINANTS RESPONSIBLE FOR VIRULENCE OF MICROBIOLOGICAL ORGANISMS

1. INTRODUCTION

Interest in the determinants of microbial pathogenicity has been revived with the goal of discovering new, more effective control, prophylactic, and therapeutic measures. Public health initiatives have controlled the worst of infectious diseases. But, in spite of major historical advances in areas such as immunology and antibiotic therapy, many problems remain. For example, no effective, broad-spectrum antiviral drug exists. There has also been a recent and alarming rise of bacterial and fungal infections in hospital patients.

An understanding of factors responsible for virulence and a basic knowledge of the mechanisms of pathogenicity could provide revolutionary approaches to disease prevention, control, and therapy; and the U.S. Army Chemical Research, Development and Engineering Center (CRDEC) thrust of generically detecting human pathogenic microorganisms would be greatly enhanced.

During the past 20 years, significant advances have occurred in biochemistry, molecular-biology, genetics, and biotechnology. Many of these technologies are ripe for studying basic mechanisms of microbial pathogenicity.

2. FACTORS INVOLVED IN VIRULENCE

The terms pathogenicity and virulence are considered synonymous with the British and American microbiological societies. Five factors are generally acknowledged to be involved in microbial pathogenicity:[1,2,3]

- Infection of mucous surfaces of the respiratory, alimentary, or urogenital tracts, or direct host entrance by skin trauma or vectors.
- Penetration of mucous surfaces to enter tissue, causing disease.
- Multiplication in the host's tissues.
- Resistance or interference with host defenses that could remove or destroy organisms (e.g., resistance to phagocytosis; immunosuppression by the AIDS virus, HTLV-III).
- Damage to the host's tissue.

If it loses its ability to carry out any one of these factors (except if organism is directly introduced), an organism's virulence could be lost.

This report reviews the methods and difficulties of investigating microbial pathogenicity using what is known of the main aspects of virulence at the molecular level. Bacteria are used as primary examples because more is known about them than other types of microbes.

The aim in investigating virulence is to identify the determinants of pathogenicity, that is, the microbial products and components responsible for the five factors (outlined above) involved in pathogenicity. The first step is to measure virulence of different strains of the chosen pathogenic species. This is usually done by finding the 50% lethal dose (LD_{50}) or an inoculum producing a skin lesion of a certain size. The second step is to identify strains of high and low virulence (either from nature or by genetic manipulation) by comparing them in the five pathogenicity requirements mentioned previously, for example, the ability to adhere to and penetrate mucosal cells, or the ability to multiply in the host's tissue, capacity to prevent ingestion by phagocytes, and the ability to damage the host's tissues. The biochemical basis is obtained by extracting and purifying the determinant[4] or manipulating genes by recombinant DNA techniques that give differences only in genes that code for a particular microbial component or product. If newly constructed strains differ in virulence and components, the gene product is the determinant.[5,6] One can test this assumption by transferring to an avirulant Escherichia coli (E. coli) two separate plasmids, one coding for K88 antigen (adherence to intestinal epithelium) and the other for enterotoxin that causes scouring in domestic animals. This then gives a strain entropathogenic for piglets.[7]

The above plan for investigating pathogenicity might present some difficulties. There could be more than one determinant because of the multifactorial nature of pathogenicity. The biochemical task is made complex because each determinant must be distinguished from others. Surface component determinants are biologically active only in situ, that is, capsular materials that interfere with phagocytosis.[8] The reattachment to the surface of a purified putative determinant, required for a final definitive biological test, is often impossible to complete. This attachment is used either on the virulent strain with the surface component removed or an avirulent strain without the component. One method to get around the problem of reattachment might be to use an antideterminant monoclonal antibody to neutralize the said biological property of the virulent strain.[8,9]

Difficulties are also found in the genetic approach. The DNA segment that codes for the putative determinant must be

the only thing biologically different in the comparison of two strains. Critical genetic analysis of genomic and plasmid DNA is required.[9] Pleitropy may occur where the gene product might not be the determinant but causes the production of other cell constituents that could be the true determinants.

Pathogenicity is measured in vivo, thus possibly creating the final difficulty. Organisms grown in vitro can be deficient in virulence determinants because of different environmental and cultural conditions; however, this can be reversed with appropriate cultural changes (e.g., the toxin responsible for death from anthrax)[4] and gonococcal resistance to complement mediated serum killing where loss of this factor in vivo was reversed by incubating with a serum fraction of small molecular weight.[8]

The above difficulties have created many gaps in our knowledge of pathogenicity and our certainty that a surface component is the determinant of one type of pathogenicity.[8] In the following presentation, each of the five factors will be used to indicate how far research has come at the observational level (i.e., when the typical biological properties have been recognized) or at the determinant stage (i.e., when a microbial product or component has been identified as either responsible for or strongly associated with the biological property).

3. BACTERIA

3.1 Infection of Mucous Surface.

Pathogenic bacteria need to move (motility) and be attracted (chemotactic) to a surface where they can adhere without being removed by lumen contents or mucociliary actions.[10-13] They need to be able to resist the host defense mechanisms found there, that is, acid or alkaline pH, bactericidal materials, and extruded phagocytes (using IgA proteases that hydrolyse IgA antibodies).[14] Pathogenic bacteria need to fight off the protective mechanisms of commensals[12,15] [i.e., occupy space needed for adherence by pathogens, use nutrients[16] (iron acquisition and tissue specificity),[17] and produce inhibitors (fatty acids, lactic acid, and H_2S)].[12,15,18] In summary, adherence is the only virulence factor at the determinant stage of research. Here, a host receptor (glycoprotein) and mannose resistant bacterial pili have been biochemically and genetically identified.[10,14,19]

3.2 Entry to the Host.

The tetanus and plague bacilli are introduced into the host directly by trauma or vectory bite. Many bacteria (e.g., staphylococci and leptospiras) that do not infect through the skin could penetrate through minute abrasions and sweat glands. If these bacteria can specifically resist host enzymes and secretions, the determinants are unknown.

Most of the methods of mucous membrane entry (the main route of entry to the host) are known only at the observational level. For example, dysentary bacilli are retained by epithelial cells, gonococci pass through the cells into the subepithelial tissue, and Salmonella typhimurium (S. typhimurium) pass through and between the cells.[8,20,21]

Determinant stage knowledge is known about Shigella flexneri (S. flexneri).[22] In this instance, entry by engulfment depends on the bacteria being alive and having a 140-M dalton plasmid that codes for protein.[8,20,23] Also, extracellular glycolipids[24] and lipoproteins[25] from S. flexneri induce engulfment by epithelial cells.

3.3 Multiplication In Vivo.

Little is known of the determinants of in vivo multiplication, but investigations show the way for future research. Avirulence (nonvirulence) can arise from inability to grow in the host's tissues.[17] Knowledge of factors that influence multiplication is limited to the restrictive influence of oxygen on the growth of anaerobic bacteria and the influence of iron supply.[16] Virulent bacteria obtain sufficient iron by secreting siderophores (iron chelating compounds) into the surrounding environment. At the same time, the virulent bacteria produces protein membrance receptors that act as receptors for the iron-bearing compound.[8,16] The effect of iron restriction on the metabolism of E. coli is indicated by a change in its transfer RNAs.[16] Brucella spp grows prolifically only in the fetal placenta, chorion, and fluids, leading to gross tissue damage and abortion. This growth is due to a stimulant (erythritol) found in the fetal tissues (not so in man or mouse) but not in the maternal tissue.[17]

3.4 Interference with Host Defense Mechanisms.

Humoral (in body fluids) and cellular (phagocytes) factors are given in many reviews and selected publications.[26-31]

3.4.1 Interference with Humoral Bactericidins.

(Late complement for gram negative bacteria: lysozyme, B lysins, and basic peptides for gram positive bacteria).[31,32] Virulent bacterial species strains are more resistant to bactericidin killing than avirulent strains (e.g., Bacillus anthracis (B. anthracis), E. coli, and meningococci.[31,32,33] Typical determinants of this resistance are capsular poly-d-glutamic acid (B. anthracis), complete lipopolysaccharide [LPS (E. coli)], and capsular polysaccharide (meningococci).[31,32,33]

3.4.2 Interference with the Action of Phagocytes.

Stages of phagocytic defense are:

- Mobilization by inflammation
- Chemotaxis towards bacteria
- Attachment and ingestion by an engulfing process (primed by opsonins that place bacteria within an intracellular vacuole, phagosome)
- Killing by oxygen-dependent system, cytoplasmic granules, and lysomes.

Bacteria can inhibit one or more of these stages. To prevent mobilization, staphylococci produce an anti-inflammatory cell wall peptidoglycan[34,35] and Treponema pallidum (T. pallidum) appears to be surrounded by an envelope that does not stimulate the inflammatory process.[32] Virulent strains of meningococci inhibit chemotaxis of phagocytes in vitro as does the cord factor of Mycobacterium tuberculosis (M. tuberculosis) and the (alpha) toxin of staphylococcus.[26,36] The capsular polysaccharides (pneumococci), M protein [Streptococcus pyogenes (S. pyogenes)], and complete LPS (S. typhimurium) are examples of some of the known chemical determinants that show the same biological activity (resistance to both attachment and ingestion by phagocytes).[8,26,31,32,34,36,37] However, E. coli attached to phagocytes are not ingested if they possess either a complete LPS or a K acid-polysaccharide.[38] Generally, the determinants work by masking specific interactions between bacterial cell-wall components and humoral opsonins.[37] However, in some cases, a nonspecific increase of surface hydrophilicity also plays a role.[38] Some bacteria grow within phagocytes by preventing phagolysosome fusion using surface sulphatides [glycol lipid sulfates (M. tuberculosis)].[26] Other bacteria resist intraphagosome killing with mycoside C, a peptidoglycolipid [Mycobacterium lepraemurium (M. lepraemurium)][39] or escape from the phagosome using outer membrane proteins (gonococci).[4,9,39] The determinant for the escape from phagosomes by M. lepraemurium is unknown.

3.4.3 Interference with the Action of Complement.

Surface or capsular materials of bacteria mask cell wall components that activate complement directly (alternate pathway) or complement after reaction with natural antibody (classical pathway).[32] Surface materials include those of mycobacterium and gonococci above plus the capsular polysaccharides of type K1 E. coli, Group B streptococci, and Group B Haemophilus influenzae (H. influenzae).[4] The elastase of Pseudomonas aeruginosa (P. aeruginosa) destroys C_1, C_3, C_5, and C_9 of the complement cascade.[26,37]

3.4.4 Interference with the Immune Response.

Bacteria suppress the action of B and T cells by various methods.[40,41] Determinants are the LPS of P. aeruginosa and the peptidoglycan polysaccharide complexes of Streptococcus.[40,41] The LPS of P. aeruginosa stimulates the production of suppressor B cells.[40] The immune response is destroyed by: (1) poor

stimulation of immune response because the determinants of virulence are bad antigen and result in incomplete neutralization and persistent infection;[9,40] (2) antigenic shift - immune response ineffective against new antigen (relapsing fever - Burrelia recurrentis);[9] (3) bacteria hide in cells (epithelial or mononuclear phagocytes) and are protected against the host defense mechanism and some injected drug (carrier state of typhoid fever, chronic tuberculosis, and brucellosis).[9] Therefore, something is known about the interference with host defense mechanisms at the determinant and observation stages that need biochemical explanations.

3.5 Damage to the Host.

3.5.1 Production of Toxins.

Domination of the multifactorial nature of virulence by Diptheria and Tetanus toxins is noteworthy.[42,43] In most cases, toxins share the equally important determinants of virulence with other factors.[44] Toxins may contain more than one component. They can inhibit protein synthesis (Diptheria, two components),[43] produce fluid loss from the gut (Cholera enterotoxin, two components),[42,43] lyse cells (Clostridium perfringens cytotoxin, one component), and cause vascular effects (Anthrax, three components).[43,45] The first component usually allows entry into the cell. Endotoxins from gram-negative bacteria (called LPS-lipopolysaccharide, pyrogen, or endotoxin) cause fever, vascular disturbances, and fetal secondary shock.[43] Except for cholera endotoxin (not released), the other endotoxins produce profound and often lethal effects.[43]

3.5.2 Damage by Immunopathological Mechanisms.

Four types of damage can occur at the observation level.[46,47,48]

- Type I. Anaphylaxis or immediate hypersensitivity caused when the IgE antibody present on mast cells reacts with antigen and releases histamine that produces vascular and respiratory effects (hay fever and asthma to lobar pneumonia).[49]

- Type II. Cytotoxic reaction occurs when antigen on host cells reacts with an antibody to prime those cells for lysis by complement or destruction by phagocytes. This occurs when viral components are inserted into the membrane of infected host cells or when host cells are similar to those of the bacteria [an autoimmune effect (Rheumatic fever)].[47,48]

- Type III. Arthus reactions occur when antigen-antibody complexes are deposited on tissue. The complexes fix complement and attract phagocytes that release their enzymes to damage tissue. This occurs in kidney damage with Proteus mirabilis and streptococcal infections.[47,48]

• Type IV. Delayed hypersensitivity reactions occur when cell-mediated immunity (CMI), stimulated by previous interaction with bacteria, mobilizes mononuclear phagocytes that release enzymes and damage tissue (Tuberculosis). Also cell-wall components from Streptococci may persist in tissue for long periods and activate complement by alternate pathways. The resulting chronic inflammation can produce arthritis.[41,49]

4. BACTERIOPHAGE

Glucosylation E. coli T shows that even bacteriophage DNA bears several modifications.[50] Not only does 5-hydroxymethylcytosine (HMC) replace cytosine, but the phage DNAs are glucosylated and methylated as well. Glucose is covalently linked through carbon 1 with the hydroxymethyl group of HMC. A major role of glucosylation is protection against host-restricting activities. T_2 normally grows on E. coli strain B and K12 and on Shigella dysenteriae (S. dysenteriae). Growth of T_2 on a specific E. coli B strain (B/40 W4597, V95, and B/3,4,7) produced altered progeny (T*2) able to grow on S. dysenteriae but not on E. coli. A single cycle of growth in the permissive host S. dysenteriae restored T*2 to normal T_2 capable of growth on E. coli and S. dysenteriae. These special E. coli strains are gal U mutants deficient in uridinediphosphoglucose (UDPG) pyrophosphorylase and thus are unable to supply UDPG for the glucosylation reaction.

5. THE PATHOGENICITY OF VIRUSES, FUNGI, AND PROTOZOA

In this instance, only the multiplication of viruses in vitro was at the determinant level.[44] The study of viral penetration and interaction with the mucous surfaces has yielded little knowledge. The determinants of the host defense mechanism against virus infection and viral interference with these defenses are largely unknown although there is much information on this subject. Also, there are many observations of damage of host cells by viral cytotoxicity and by immuno-pathogenicity, but biochemical studies have just begun.[44] Fungal toxins (mycotoxins) are usually products of fungal growth at extreme or unusual conditions.[51] Once again, pathogenicity is only at the observation level.

6. ENZYMES

Pathogenic bacteria secrete exoenzymes that interact with various components of the infected host's tissue, but the actual virulence mechanisms are varied.[52] Bacterial enzymes may release host nutrients for further bacterial growth or the production of toxic secondary metabolites. Also, enzymes may act as toxins that interfere with necessary host functions, (e.g., ADP ribosylating enzymes produced by Corynebacterium diptheria, P. aeruginosa, E. coli, and Vibrio cholera. Other enzymes (proteases) overtly destroy host tissue or neutralize

the host defense mechanism (P. aeruginosa). Streptococcus, Neisseria, and Haemophilus species produce enzymes that have a narrow substrate specificity against the secretory immunoglobin IgA, an important defense mechanism. The association between elaboration of specific exoenzyme and virulence is sometimes very clear. In other cases, the relationship is speculative.

The correlation between plasmids and protease enzyme production is summed up as follows:[53] "There is apparently no correlation between IgA production and plasmid carriage. A plasmid-free strain of N. gonorrhoeae retained the ability to produce IgA protease.[54] In addition, several isolates of H. influenzae and S. pneumoniae, including a protease-negative isolate of H. influenzae and several strains producing 'double' enzymes, were examined for the presence of plasmids. No correlation was found between the presence of extrachromosomal DNA and IgA protease production."[55]

7. CONCLUSIONS

This review of the knowledge of the mechanism of pathogenicity shows that some of the determinants of all five factors involved in the aspects of bacterial pathogenicity have been identified. For other microbes, only the multiplication of viruses in vivo was at the determinant level. Once we have found that the determinant is a gene product, we have two avenues to detect virulence: (1) the determinant, usually a surface component, or (2) the gene found on chromosomes, plasmids, or phages. When considering genetic engineering, where genes are moved from one organism to another (e.g., by using plasmids), a test for virulent genes is needed. This may be done with a gene probe from a virulent microbe, usually a labeled single-stranded deoxyribonucleic acid, that will recombine only with single-stranded nucleic acid from similar virulent genes. Gene probes can detect a gamut of genes from a strain or serological type of single species to large groups consisting of Families or Orders of microbes. Novel detection options for gene probes may include use of wave guides, fiber optics, tissue or chemical receptors, enzyme assays, and/or labeled colorimetric residues.

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